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Antigenic role of the endosymbionts of filarial nematodes: IgG response against the *Wolbachia* surface protein in cats infected with *Dirofilaria immitis*

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Filarial nematodes harbour intracellular endosymbiotic bacteria, which have been assigned to the genus *Wolbachia*. These bacteria appear to play an important role in the pathogenesis of filarial diseases through their lipopolysaccharides. In view of the presence of *Wolbachia* endosymbionts in the body of filarial nematodes, one might also expect that proteins from these bacteria play an antigenic role in humans and animals affected by filariases. To test this hypothesis, we produced in recombinant form the surface protein WSP and a portion of the cell-cycle protein FTSZ from the *Wolbachia* of *Dirofilaria immitis*. Western immunoblot assays were then performed using cat sera to test the immunogenicity of these proteins. Sera were collected from owners' cats, which were either sero-negative or sero-positive for *D. immitis* and from cats before and after experimental infection with *D. immitis*. FTSZ was recognized in Western blots by sera from both positive and negative cats and from both uninfected and experimentally infected cats. WSP was recognized only by sera from positive cats and from cats experimentally infected with *D. immitis*; this protein was not recognized by sera from negative cats and from cats before experimental infection with *D. immitis*. The results of Western blot assays on WSP thus support the hypothesis that infection with filarial nematodes induces the production of antibodies against *Wolbachia* proteins.

Keywords: filariasis; immunology; *Wolbachia*; heartworm; *Wolbachia* surface protein

1. INTRODUCTION

Filariasis is still a major health problem in humans and animals. The main filariasis agents, *Brugia malayi*, *Onchocerca volvulus* and *Wuchereria bancrofti*, infect an estimated 150 million people in tropical countries (Ottesen 1992, 1995). The heartworm disease of dogs and cats is caused by another filarial nematode, *Dirofilaria immitis* (Boreham & Atwell 1988). Pathogenesis of filarial diseases is associated with a range of inflammatory conditions (Ottesen 1992; 1995). Identification of filarial molecules responsible for pathogenic immune responses and immunomodulation has been a major goal of experimental studies on filariasis (see, for example, Rao *et al.* 1999). Recent breakthroughs in this research area have come from investigations focused on the bacterial endosymbionts of filarial nematodes (Taylor *et al.* 2000), which had previously been identified as *Wolbachia* (Sironi *et al.* 1995; Bandi *et al.* 1998). *Wolbachia* endosymbiotic bacteria are widespread in filarial nematodes. Based on the available data, there is no polymorphism for the presence of *Wolbachia* in a given species of filaria: where *Wolbachia* has been shown to be present, all individuals examined have been shown to be infected (Bandi *et al.* 1998; Taylor & Hoerauf 1999). *Wolbachia* thus appears to be a stable component of the body of filarial nematodes, at least in the major pathogens of humans and animals. These bacteria appear necessary for the development, reproduction and long-term survival of filarial nematodes and may thus represent a target for the control of filariasis (Genchi *et al.* 1998*a*; Bandi *et al.* 1999; Hoerauf *et al.* 1999, 2000; McCall *et al.* 1999; Langworthy *et al.* 2000). *Wolbachia* endosymbionts also appear to play an important role in the inflammatory pathogenesis of lymphatic filariasis through their lipopolysaccharides (Taylor *et al.* 2000). It has also been suggested that these endosymbionts could play an antigenic role (Kozek 1977; Kozek & Figueroa 1977; Bandi *et al.* 1998), but so far no experimental evidence has been presented in support of this idea. In order to address this issue, we decided to test two proteins of *Wolbachia* by Western blotting with sera from infected and uninfected animals.

We focused our experimental work on *D. immitis*. Two proteins from the *Wolbachia* of *D. immitis*—the *Wolbachia* surface protein (WSP; Braig *et al.* 1998) and the cell-cycle protein FTSZ (Osteryann & Vierling 1995)—were produced in recombinant form. To test the immunogenicity of these proteins we used pre- and post-infection sera from cats experimentally infected with *D. immitis* and sera from owners' cats, which were either sero-positive or sero-negative for *D. immitis* infection.

The genes coding for WSP and FTSZ have been sequenced for the *Wolbachia* endosymbionts of several species of filariae (Bandi *et al.* 1998; Bazzocchi *et al.* 2000; Casiraghi *et al.* 2001). FTSZ and proteins homologous to WSP are known to be antigenic in other bacteria (Pademalayam *et al.* 1997; Ohashi *et al.* 1998). In

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particular, WSP homologues from *Cowdria* spp., *Ehrlichia* spp. and *Anaplasma marginale* have been shown to be major antigens (Ohashi *et al.* 1998). These proteins show a high level of sequence divergence among different bacterial genera (Ohashi *et al.* 1998) and are thus expected to exhibit a low level of cross-reactivity in serological tests. FTSZ has also been shown to play a major antigenic role in *Bartonella bacilliformis* (Pademalayam *et al.* 1997). However, since FTSZ sequences are quite conserved among distantly related bacteria (Osteryang & Vierling 1995), cross-reactivity in serological tests might be expected when using this protein as an antigen. WSP and FTSZ are thus good candidate antigens for the *Wolbachia* of filarial nematodes, even though they are expected to show different levels of specificity.

Cats are known to develop a strong IgG response after infection with *D. immitis* (Prieto *et al.* 1997) and immunopathological phenomena are thought to play an important role in the pathogenesis of filariasis in these animals (McCall *et al.* 1994). In areas where *D. immitis* is endemic, disease prevalence is lower in cats than in dogs. Sero-prevalence is also lower in cats (Guerrero *et al.* 1992; Genchi *et al.* 1998*b*); this permits experiments to be carried out on sera samples from sero-negative and sero-positive cats collected from the same location. On the other hand, dogs sero-negative for *D. immitis* are expected to be infrequent in endemic areas. For these reasons, the cat appears to be a more suitable model for testing the immunogenicity of components from *D. immitis*.

2. MATERIAL AND METHODS

(a) *Over-expression and purification of the mature WSP*

The DNA fragment coding for the mature WSP protein of the *Wolbachia* of *D. immitis* (accession number AJ252062; Bazzocchi *et al.* 2000) was amplified by PCR. The forward primer was 5′-ACGTCCGAGCGGCGGATCCGGTCCTATTGGTCCA (corresponding to amino-acid positions 1–5 of the mature protein). The reverse primer was 5′-ACGTCGGTGCGGCCTGCAG-CTAAAAATTAAACAT (corresponding to amino-acid positions 209–212 of the mature protein plus the stop codon). These primers include at their 5′-ends restriction sites (underlined) for ligation into the PDS56 expression vector (Roche Molecular Biochemicals, Rotkreuz, Switzerland): forward primer, *Bam*HI; reverse primer, *Pst* I. The recombinant plasmid obtained after ligation was named *wsp*-PDS56.

The expected fusion protein includes mature WSP (lacking the signal sequence: amino acids 1–24 of the full-length protein) and nine additional amino acids encoded by the vector at the N-terminal (MHHHHHHGS). The six histidines encoded by the vector (His tag) have the function of enabling the fusion protein to be purified by affinity chromatography. Plasmid *wsp*-PDS56 was amplified in *Escherichia coli* M15pRep4 and, after sequencing a sample of clones using ABI technology, a clone with the correct gene sequence was selected. *E. coli* M15pRep4 cells containing *wsp*-PDS56 were grown at 37 °C in 50 ml Luria–Bertani (LB) medium containing ampicillin (100 μg ml^{-1}) and kanamicin (25 μg ml^{-1}) to an A_{600} of 0.5 before induction of recombinant WSP expression by addition of 1 mM isopropyl b-D-thiogalactopyranoside. Cells were harvested after 2 h by centrifugation at 4000 *g* for 20 min. Collected cells were disrupted by freeze-thawing and sonication, and recombinant WSP was collected as an insoluble aggregate by centrifugation at 10 000 *g* for 10 min.

The recombinant WSP was solubilized in 0.1 M NaH_2PO_4, 0.01 M Tris HCl, 6 M urea (pH 8.0) and was purified by high-performance liquid chromatography (Poros MC/M 2.1 × 30 mm) using Cu^{2+} as a ligand. The protein was then eluted in one step by adding 100 mM imidazole in solubilization/loading buffer.

(b) *Over-expression and purification of a portion of the FTSZ protein*

A DNA fragment coding for a 336 amino-acid segment of the FTSZ protein from *Wolbachia* of *D. immitis* (accession number AJ010272; Bandi *et al.* 1998) was amplified by PCR. The forward primer was 5′-TACGGAATTCCAAGCGCTAGAAAAGTCAT-TG. The reverse primer was 5′-TATACAAGCTTTCAATAA-ATGTTATTATTCCATC. These primers are located at the 5′- and 3′-ends of the partial *ftsZ* gene sequence available for the *Wolbachia* of *D. immitis*. Locations of these primers on the full-length gene sequence of the *Wolbachia* of *Drosophila melanoganster* (accession number X71906) are 151–171 and 1145–1164. These primers include at their 5′-ends restriction sites (underlined) for ligation into the pMAL-C^{TM} expression vector (New England Biolabs, Beverly, MA, USA): forward primer, *Eco*RI; reverse primer, *Hind*III. Primer reverse also includes a stop codon. The recombinant plasmid obtained after ligation was named *ftsZ*-pMAL-C.

The expected fusion protein includes a portion of FTSZ and the maltose binding protein (MBP) encoded by the vector. The MBP has the function of enabling the fusion protein to be purified. Plasmid *ftsZ*-pMAL-C was amplified in *E. coli* TB1 and, after sequencing a sample of clones using ABI technology, a clone with the correct gene sequence was selected. *E. coli* TB1 cells including *ftsZ*-pMAL-C were grown at 37 °C in 200 ml LB medium containing ampicillin (50 μg ml^{-1}) to an A_{600} of 0.5. Expression of recombinant protein in *E. coli* was induced by the addition of 0.3 mM of IPTG at the mid-log phase of growth. Cells were harvested after 3 h. Purification of the fusion protein was then performed using the pMAL^{TM} Protein Fusion and Purification System (New England Biolabs) according to the manufacturer's instructions.

(c) *Cat sera*

Sera from three cats (NYI, ORI, and CA4) experimentally infected with *D. immitis* were provided by the TRS Laboratories, Inc. (Athens, GA, USA). Pre- and one-, four- and nine-month post-infection sera were collected from the same three animals. These cats had been bitten by mosquitoes infected with *D. immitis*. Adult heartworms were recovered from all these cats: NYI, seven worms (three males, four females); ORI, 18 worms (11 males, seven females); CA4, five worms (one male, four females).

One hundred and fifteen cat sera were also obtained from owners' cats living in a restricted area of the Po River Valley (in the surroundings of Alessandria). In this area, *D. immitis* infection is endemic. For the diagnosis of *D. immitis* infection, two different antibody-capture tests were used. A first screening was effected using the Solo Step Filaria Gatto^{TM} diagnostic kit (Heska, Fort Collins, CO, USA). Based on the results of this screening, we chose a subsample of 54 sera. These sera were then examined using an enzyme-linked immunosorbent assay (ELISA) test based on the detection of antibodies against excretory/secretory heartworm antigens (Prieto *et al.* 1997). We finally chose two groups of sera: the first group included 20 sera

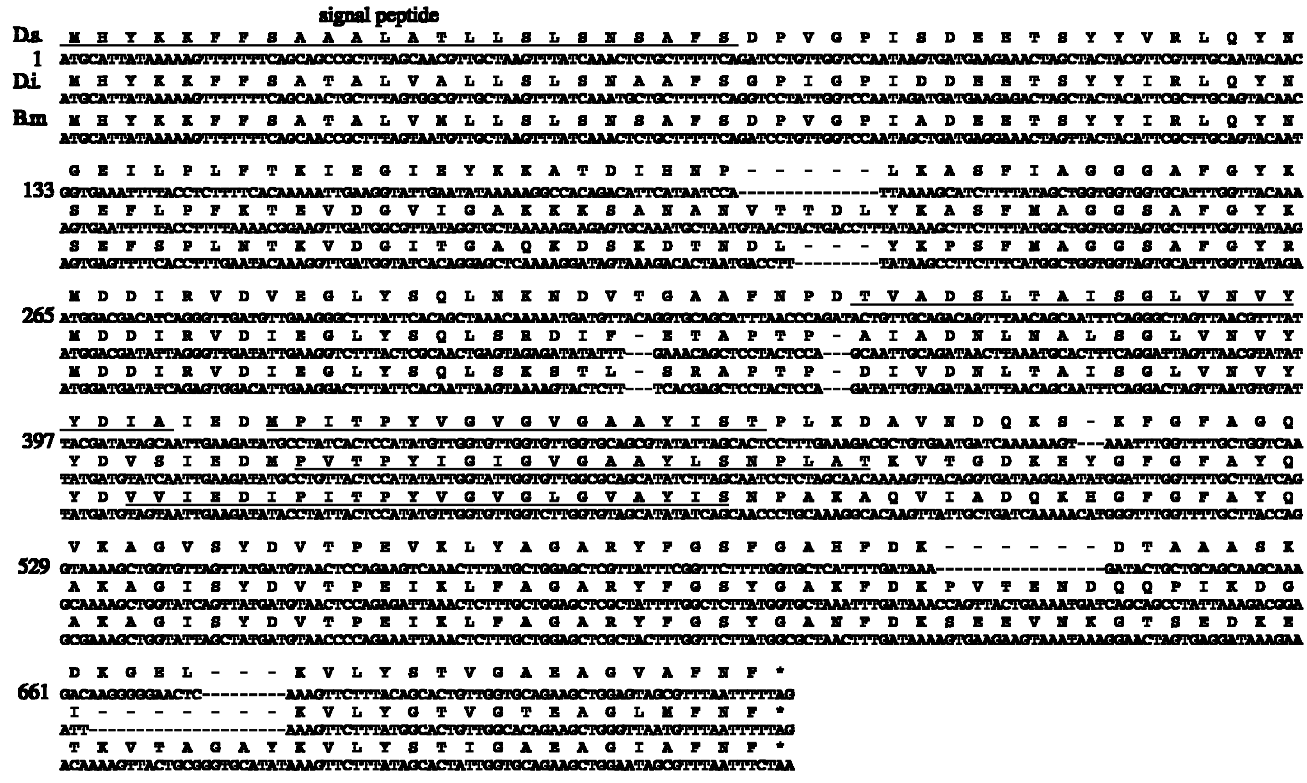

Figure 1. Alignment of *wsp* genes and corresponding amino-acid sequences from the *Wolbachia* of *Drosophila simulans* (D.s.; accession number AF020070), *Dirofilaria immitis* (D.i.; accession number AJ252062) and *Brugia malayi* (B.m.; accession number AJ252061). The signal peptide is indicated and shows perfect overlap in the three sequences (underlined amino acids on D.s. sequence). Transmembrane domains are indicated for both nematode- and arthropod-derived sequences (underlined amino acids). Stop codons are indicated by an asterisk.

that were sero-positive for *D. immitis* with both tests; the second group included 20 sera that were sero-negative with both tests.

(d) *Western blot analysis*

The purified recombinant proteins quantified using the Bio-Rad Protein Assay™ (Bio-Rad, Hercules, CA, USA) were used for Western immunoblot analysis. Fifty nanograms of the recombinant WSP and 100 ng of the recombinant FTSZ were separated by 12% SDS–polyacrylamide gel electrophoresis (PAGE) and electro-transferred on to a nitrocellulose membrane. Blots were blocked for 20 min with PBS containing 0.1% Tween 20 and 2% milk. Membranes were incubated for 1 h at room temperature with cat sera at 1:100 dilution and then incubated for 45 min with peroxidase-conjugated anti-cat IgG (ICN, Irvine, CA, USA) at a 1:5000 dilution. The peroxidase-positive bands were detected using the opti-4CN™ substrate kit (Bio-Rad). Western blot analysis was also performed on the MBP using sera from pre- and post-infection (nine months) cats.

3. RESULTS AND DISCUSSION

The complete *wsp* gene sequence of the *Wolbachia* of *D. immitis* contains an open reading frame (ORF) of 708 bp coding for 236 amino acids (figure 1). The 24 amino-acid sequence at the N-terminal of the WSP is likely to be the signal peptide of the protein. In addition, analysis of the WSP sequence of the *Wolbachia* of *D. immitis* predicts at least one transmembrane domain, which overlaps the second transmembrane domain of arthropod WSP (Bazzocchi *et al.* 2000; see also accession ds41508 in the EMBL alignment database). Similarly, in the other full-length gene sequence available for a filarial *Wolbachia* (from *B. malayi*; Bazzocchi *et al.* 2000), a signal peptide and a transmembrane domain are predicted (figure 1). Primers for cloning and expressing *wsp* from the *Wolbachia* of *D. immitis* were thus designed in order to produce only the mature WSP (i.e. excluding the signal peptide; molecular weight (MW): 23 kDa).

The partial *ftsZ* gene sequence available for the *Wolbachia* of *D. immitis* (accession number AJ010272; Bandi *et al.* 1998) contains an ORF of 1008 bp coding for 336 amino acids (MW: 36 kDa). Primers for cloning and expressing the *ftsZ* of the *Wolbachia* of *D. immitis* were designed in order to produce this whole fragment.

Both WSP and FTSZ were expressed as fusion proteins: WSP included a His tag and FTSZ included the MBP (Nygren *et al.* 1994). After purification, SDS–PAGE showed that the recombinant WSP and FTSZ were free of contaminating *E. coli* proteins (not shown). The MW of the recombinant WSP and FTSZ proteins are 24 kDa (His tag: 1 kDa; WSP: 23 kDa) and 76 kDa (MBP: 40 kDa; FTSZ: 36 kDa), respectively. The entire fusion proteins were used in Western blot experiments. Since the short His motif is not expected to alter the results of immunoblot assays, fusion proteins including the His tag are commonly used in Western blot experiments (see, for example, Webb *et al.* 1998). Similarly, MBP is not expected to alter immunoblot results, and has also been used as a carrier protein for filarial antigens in Western blot and ELISA assays using cat sera (Mejia *et al.* 1994; Hong *et al.*

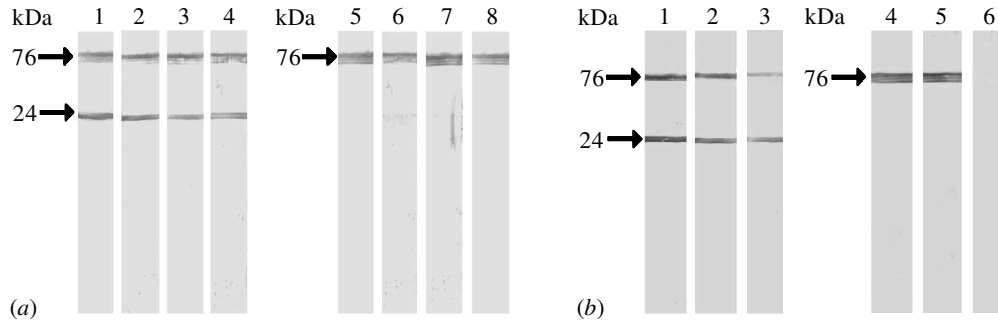

Figure 2. Examples of Western blot analysis on recombinant WSP (24 kDa) and FTSZ (76 kDa). (*a*) Western blot using sera from owners' cats sero-positive (lanes 1–4) and sero-negative (lanes 5–8) for *D. immitis*. (*b*) Western blot using sera from three cats collected nine months after experimental infection with *D. immitis* (lanes 1–3) and sera from the same three cats collected before experimental infection (lanes 4–6); lanes 1 and 4, cat NYI; lanes 2 and 5, cat CA4; lanes 3 and 6, cat ORI.

1996). However, we decided to test whether this protein is recognized by cat sera. Western blot experiments on the MBP using sera from both infected and uninfected cats did not produce detectable bands (not shown).

Western blot experiments on the purified WSP and FTSZ were first done using sera from owners' cats (figure 2*a*). Sera from the 20 cats that were sero-positive for *D. immitis* infection generated two bands of 24 and 76 kDa, corresponding to the expected MW of the recombinant WSP and FTSZ proteins, respectively (figure 2*a*, lanes 1–4). Sera from the 20 sero-negative cats produced only one band of 76 kDa (figure 2*a*, lanes 5–8). This indicates that IgG against WSP were not present in these heartworm-negative sera. We emphasize that sera from sero-positive and sero-negative cats were collected in the same location. The presence of IgG against WSP in sero-positive cats is thus likely to be related to *D. immitis* infection as opposed to factors associated with the collection area and/or cat population.

Further Western blot assays were immunostained using sera from three experimentally infected cats (figure 2*b*). Nine-month post-infection sera generated bands of 24 and 76 kDa (figure 2*b*, lanes 1–3). However, one of the bands at 76 kDa was quite faint. Pre-infection sera from the same three cats were also tested. These three sera did not produce any bands at 24 kDa. Two of them produced a band of 76 kDa (figure 2*b*, lanes 4–6). Western blotting on WSP was also done using sera collected at one and four months post-infection from the same cats (figure 3). As in the previous experiment, bands at 24 kDa were strongly visible using the nine-month post-infection sera. These bands were also detectable using the four-month post-infection sera, while one-month post-infection sera produced only very faint bands (not visible after scanning of the membranes).

The results of our Western blot experiments show that WSP is recognized by sera of infected cats. The recognition patterns obtained using experimentally infected cats clearly showed that, after infection with *D. immitis*, the animals developed an immune response against WSP (figures 2 and 3). While further experiments are necessary to describe the dynamics of this response after the infection, the pattern displayed by the sera from the one-, four- and nine-month post-infection animals (figure 3) is comparable to that observed in dogs and cats using both

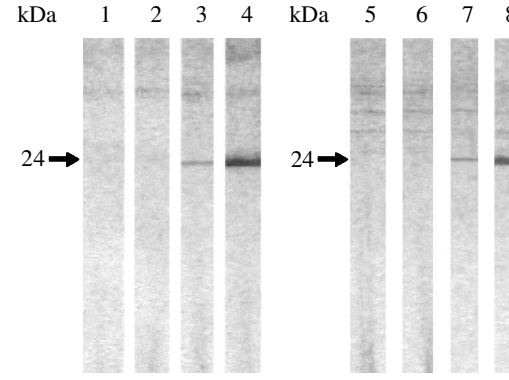

Figure 3. IgG response of cats to WSP during the course of infection with *D. immitis*. Example of Western blots analysis on recombinant WSP (24 kDa) using sera collected from two different cats (lanes 1–3, NYI; lanes 4–6, CA4). Lanes 1 and 5, sera collected before the experimental infection; lanes 2 and 6, sera collected one month after infection; lanes 3 and 7, sera collected four months after infection; lanes 4 and 8, sera collected nine months after infection.

recombinant proteins or purified antigens of *D. immitis* (Mejia *et al.* 1994; Hong *et al.* 1996; Prieto *et al.* 1999).

Since all but one of the sera used in this study recognized the recombinant FTSZ, we are unable to derive any conclusion regarding the possibility that the FTSZ of *Wolbachia* determines an IgG response in cats. One possible explanation for the fact that sera from both infected and uninfected cats recognized FTSZ is that this protein is a common component of prokaryotic cells and contains amino-acid motifs that are conserved even among distantly related bacterial species. Some of these motifs are also conserved in eukaryotic tubulin (Osteryang & Vierling 1995). It is thus possible that cats develop an IgG response against the bacterial FTSZs they meet during their life and we may expect to observe antibody cross-reactivity among the FTSZ of *Wolbachia* and those of other bacteria. Among the cat sera we tested, only one from a pre-infection experimental animal did not recognize the FTSZ of *Wolbachia*. That a faint band was produced by the serum of this cat after infection with *D. immitis* seems to suggest that this animal had no anti-FTSZ antibodies before infection and then developed a weak response against this protein.

Although the experiments using FTSZ did not allow us to demonstrate any specific immune response against this *Wolbachia* protein, they are useful for evaluating the results obtained using WSP. Since sera from most of the pre-infection experimental cats and from the sero-negative owners' cats recognize FTSZ, this protein appears to be a useful control for the quality of these sera. In addition, since the intensity of FTSZ bands using most of the sera from pre- and post-infection animals (or from sero-positive and sero-negative animals) were comparable, it is unlikely that Western blot recognition of WSP after infection with *D. immitis* (or by sero-positive cats) was determined by a non-specific increase of IgG serum levels.

The discovery of intracellular bacteria (later shown to be *Wolbachia*) in the body of filarial nematodes led the authors to hypothesize that these bacteria were implicated in the immunology and pathogenesis of filarial diseases (McLaren *et al.* 1975; Kozek 1977; Kozek & Figueroa 1977; Bandi *et al.* 1998). Until recently, however, no experimental studies have attempted to falsify this hypothesis. It has recently been shown that the lipopolysaccharides (or endotoxins) of *Wolbachia* may be major determinants of inflammatory pathogenesis in lymphatic filariasis (Taylor *et al.* 2000), but the possibility that *Wolbachia* plays an antigenic role has not yet been addressed. Even though the latter appears a reasonable possibility, questions about the capacity of *Wolbachia* endosymbionts to actually stimulate an immune response might be raised. For example, there are the questions of whether filarial nematodes harbour enough *Wolbachia* (or enough *Wolbachia* antigens) to stimulate an immune response and of whether enough *Wolbachia* antigens are released during the development or at the death of filarial nematodes. The results described here represent the first experimental evidence, to our knowledge, for the idea that infection with a filarial nematode harbouring *Wolbachia* leads to an immune response against *Wolbachia* proteins. The possibility that *Wolbachia* proteins play a role in inflammatory pathogenesis is thus worthy of investigation. The approaches which have been widely used to examine the proinflammatory or immunomodulatory effects of nematode proteins could be used on WSP and other *Wolbachia* proteins (see, for example, Garraud *et al.* 1995; Rao *et al.* 1999).

Cats are not easily infected by *D. immitis*. In experimentally infected cats, the number of third-stage larvae that develop into adult worms is limited (McCall *et al.* 1994). The presence of IgG against WSP after experimental infection with *D. immitis* is possibly due to the death of larvae during their development with the consequent release of somatic antigens, including proteins from *Wolbachia*. However, it is also possible that *Wolbachia* bacteria (or their proteins) are released from filarial nematodes during their normal development and survival. Notably, antibodies against WSP have also been detected in dogs sero-positive for *D. immitis* (C. Genchi, unpublished observation). This suggests that *Wolbachia* proteins induce an immune response also in a host more susceptible to *D. immitis* infection.

Diagnosis of heartworm infection in cats relies on the use of methods for the detection of specific antibodies. Infected cats are frequently amicrofilaraemic and the presence of circulating antigens in this host is also infrequent (occult infections). Based on our results, WSP appears to be potentially useful for the diagnosis of dirofilariasis in cats. Our study shows that proteins of *Wolbachia* should be taken into account for the development of diagnostic methods in both animals and humans. Further studies are, of course, necessary to confirm whether *Wolbachia* is always present in infected filarial species. So far we have examined more than 50 samples of *D. immitis* collected worldwide: all have been found to harbour *Wolbachia* (Bandi *et al.* 1998; C. Bazzocchi and C. Bandi, unpublished observations).

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